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Art. XXIII.—*On Bitter Pit and Sensitivity of Apples to Poison.*

By ALFRED J. EWART, D.Sc., Ph.D.

(Professor of Botany and Plant Physiology in the Melbourne University).

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In 1913 Rothera and Greenwood made a direct attempt to test the poisoning theory of Bitter Pit, on the line that if the non-solution of the starch grains usually shown in Bitter Pit tissue is due to the action of the poison, a diastase solution after contact with Bitter Pit tissue should have its diastatic activity retarded. They stated, however, that with malt diastase and Taka diastase an accelerating action was shown. In the Proceedings of the Royal Society of Victoria, Vol. 26, p. 233, I showed, however, that they had overlooked the influence of the presence of tannic acid, and that even a short contact of 10 c.c. of 1 % Taka diastase with 20 grams of pounded apple pulp distinctly retarded the diastatic activity of the filtered extract. It is well known that tannic acid retards diastatic action, and this was supposed to be due to a direct action on the diastase. I showed, however, that tannic acid, even when dilute, precipitates starch from a watery solution, and thence concluded that the action was rather on the starch than on the diastase. This is borne out by the fact that the precipitating action is less pronounced at high temperatures, and that under these circumstances the retarding action of the tannic acid is also relatively less pronounced.

In replying to my criticism, Rothera and Breidahl reaffirm the existence of an accelerating action. This might be obtained under the following conditions :—(1) If a resistant and very active diastase such as Taka diastase is used in relatively large amount. (2) If the tests are made at high temperatures. (3) If dry bitter pit pulp in which the tannic acid has been oxidised is compared with fresh pulp rich in tannic acid.

I found that using equal volumes of 1 % taka diastase or malt diastase, of 1 % starch solution, and of tannic acid, the latter retarded the hydrolysis of starch down to concentrations of 0.005 to

0.001 % at 28° and 35° C. With larger amounts of tannic acid an apparent acceleration may be shown, but this is simply due to the tannic acid condensing and precipitating the starch, so that the liquid above gives yellow with iodine. If the whole of the remaining starch is precipitated with excess of tannic acid, filtered, dried and weighed, the control always contains less starch than the tube with tannic acid. To get the full retarding action, the diastase extract must contain no proteids capable of combining with and removing the tannic acid, and for this reason filtered malt diastase is more sensitive to the presence of tannic acid than unfiltered malt diastase. When the diastase extract is free from proteids capable of removing tannic acid, the retarding action is probably entirely due to the action of the tannic acid on the starch and not to an action on the diastase. In addition, I was able to show that dilutions of metallic poisons unable to destroy either oxidase or diastase were still poisonous to the living protoplasm of the apple and potato when applied externally. It is, in fact, a fairly general rule that enzymes are a little more resistant to dry and moist heat and to poisons than the protoplasm of the cells containing them, and hence the diastase method will only detect a poison when present in relatively large amount and in soluble form, and even then only when nothing else which affects diastatic action is present in the tissue.

At the September meeting of the Royal Society of Victoria, Rothera, together with Miss Kincaid and Miss Jackson, advanced a criticism of my work on the sensitivity of apples to poison. They stated that the poisoning effects obtained by me were not due to the poisons used at all, but to the action of the distilled water to which the apple pulp was exposed at the points where the cuticle had been removed. They based this conclusion on the following statements: —(1) Prepared apples floated on distilled water developed brown pits beneath the points from which the "cuticle" had been removed. (2) In isosmotic (isotonic) solutions of sodium chloride (2.5 % and upwards) to which poisonous solutions were added, no brown pits developed. (3) Peeled apple pulp floated on distilled water slowly turns brown, but remains colourless when floated in apple sap. They conclude, therefore, that in (2) and (3) the pulp cells are under normal osmotic conditions, and the pulp cells remain living, and that in (1) they are under abnormal osmotic conditions and therefore die. As a matter of fact the reverse is the case. No plant cell provided with a cell-wall can grow in a medium isosmotic

with the cell-sap. The essential feature of a typical plant cell as compared with an animal cell is that it avoids isosmotic conditions, and spends its whole life not quite in distilled water, but in a very dilute solution containing usually not more than one gram of dissolved solids in 1 to 2000 c.c. of water. This water saturates the cell-wall, and the strong solution inside the cell presses the semi-permeable protoplasm against the cell-wall, and stretches the latter until its distension balances the surplus osmotic energy of the cell sap when a condition of the hydrostatic equilibrium is reached. If the cell is now placed in an isosmotic solution of an impermeable salt, the cell wall is no longer stretched, no growth is possible, and the cell is in an entirely abnormal condition.

Scarlet Nonpareil apples of approximately the same shape and size were selected, only varying a few grams from 800 grams weight. After removing the cuticle from 15 points in areas of as nearly as possible 1 mm. diameter, the apples were weighed and floated in water and 2.5 salt solution for 1 week. The first apple gained 0.6 gram per cent. in weight, the second 0.057 grams. In a second experiment the calyx and stalk were covered with paraffin. In distilled water the apple absorbed 0.45 c.c. of water per 100 grams, in the salt solution it lost 0.03 c.c. In 0.5 % and 1.5 % solutions of sodum chloride distinct gains of weight were shown, but always less in the 1.5 % as compared with the 0.5 % solution, and in the latter as compared with distilled water, provided that the skin of the apples was without injury or crack so that water could enter only at the prepared points.

The amount of absorption will depend largely upon whether the osmotic pressure of the pulp cells is or is not fully satisfied in the apple before it is immersed in water. Hence it is important to use apples fresh from cool storage, in which the loss by transpiration has been slight. In the tests with very dilute poisonous solutions, a little of the solution is drawn into the apple at special points where the poisonous action is localised, in addition to the poison reaching the surface by diffusion. In one experiment with 1 per 100,000 copper sulphate, 0.4 gram of the solution was absorbed and 2.4 grams of tissue were poisoned, so that to poison 1 gram of the pulp cells required at least one millionth of a gram of anhydrous copper sulphate.

Although prepared apples soaked in 1.5 % and 0.5 % salt solutions absorb appreciable quantities of the solution, the prepared spots show at first sight no signs of poisoning and remain colourless

or nearly so, instead of turning brown. If fresh apple pulp is pounded up with 0.5 to 2.5 % solutions of sodium chloride, it does not turn brown, although the cells are completely killed. The pulp gives even after some hours a faint blue with guiacum, a strong one with guiacum and hydrogen peroxide, and a fairly rapid reaction with ursol tartrate. Salt, therefore, prevents the oxidation of tannic acid by apple oxidase without destroying the latter, and it is a sensitiser to the oxidase action on guiacum, which normally only turns blue with guiacum in the presence of hydrogen peroxide. I have already given specific instances of many similar specific "antioxidase" and "sensitiser" reactions, and have shown that the presence of salt affects various of the colour reactions of tannic acid, including its reaction with ferric chloride.

Owing to the action of salt in preventing browning it is difficult to determine its poisonous action. So far as can be judged by microscopic examination of the cells beneath the prepared spots where the salt solution is absorbed, it appears to belong to the class of almost non-poisonous salts as compared with mercury and copper salts, and to be less poisonous than potassium salts. With strong solutions osmotic injury is caused, but this is mainly confined to the surfaces of the prepared spots.

In regard to the statement that brown pits developed in apples floated on distilled water beneath the prepared spots from which the cuticle had been removed, I was fortunately able to examine subsequently the apples in question and to see that not only the cuticle but also the epidermis and hypodermis had been removed right down to the pulp tissue. The importance of not removing these layers is that they form continuous layers of cells without air spaces (except at the lenticels), and hence prevent the invasion of micro-organisms, which takes place very rapidly in water, particularly if any of the pulp cells have been injured or cut, and is soon followed by an invasion of fungal hyphae.

The browning of peeled pulp floated in distilled water is usually due to the action of micro-organisms. They can be seen in a few hours, and if the water is previously sterilised, all possible antiseptic precautions taken and the peeling done with a sharp razor, the browning of the pulp is very slow. Peeled pulp will remain fresh and living under kerosene for as long as 2 to 3 weeks, showing that the death of the pulp is not due to asphyxiation by drowning. In fact, apples remain living for some weeks in an atmosphere of nitrogen or hydrogen.

When immersed in its own sap living pulp usually remains unbrowned for some time if the sap has been previously sterilised by boiling. The soluble matters in this sap are impermeable to the living protoplasm, just as they are when inside the cell. No penetration therefore takes place, and the tannic and other acids of the sap prevent or retard the development of bacteria. Such pulp is, however, readily invaded by fungal hypae.

In all cases, for a poisoning effect to be exercised the poison must be able either to penetrate the protoplasm or to injure its ectoplasmic membrane. A curious point worth noting here is that the protoplasm of the pulp cells is, as one might expect, resistant to tannic acid. This is probably due to the formation of an impermeable coagulation film on the surface of the ectoplasmic membrane, such as must exist normally on the endoplasmic membrane. This membrane appears to increase the impermeability of the protoplasm, especially to organic acids (malic, citric, tartaric, oxalic), and hence in the presence of tannic acid externally applied, solutions of these acids are only poisonous in considerably increased concentration. This may explain the remarkable effectiveness with which the delicate pulp cells retain their sugary and acid contents, although some of the acids when applied externally are poisonous.

It is perhaps hardly necessary to say that in my own work these possibilities of error were detected early. Every experiment was done with a control in distilled water. These controls were unaffected, and, in fact, properly prepared apples can be kept almost as well floating in distilled water as ordinary apples can be kept in air. In addition the results were throughout consistent—i.e., with increasing dilution less and less poisoning effect was exercised. In order to settle this matter finally, I arranged to perform these experiments before a committee consisting of Dr. Hall, President of the Royal Society, Professor Osborne, Dr. Rothera, Miss Kincaid and Miss Jackson. All the apples were prepared by me. In the whole series about 260 removals of the cuticle from usually 10 to 12 points in each apple took place. In about 5 or 6 cases the cut was a little below the cuticle. Part of the solutions (series A) were prepared by myself, part (series B) by Dr. Rothera, Miss Kincaid and Miss Jackson. The apples were placed in the solutions by one of the three foregoing. They were kept in a locked cupboard by Dr. Hall and inspected jointly after 3 and 7 days' immersion. The solutions were then poured away, the apples and cylinders washed with distilled water, left for a week

in moist air and examined by the members of the committee. The average temperature was 15°-18° C. Dr. Rothera brought Gravenstein apples for testing. I preferred Yates' Pippin, which is a hard-fleshed, resistant apple, much less sensitive to poisons but always unaffected by distilled water if properly prepared.

The results of the test are as follows :—

Series A.—Yates' Pippin.

Controls.

Distilled water.

(1) No pits, browning or signs of poisoning on any of the prepared spots.

(2) No pits, browning or signs of poisoning on any of the prepared spots.

Copper Sulphate.

(3) 1 per 100,000. Well-defined brown pits 1-2 mm. deep on all prepared spots.

Lead Nitrate.

(4) 1 per 100,000. Doubtful.[1]

Mercuric Chloride.

(5) 1 per 10,000. Large pits 1-3 mm. deep.

(6) 1 per 100,000. Small brown pits 1-2 mm. deep on all the prepared spots.

(7) 1 per 1,000,000. Doubtful.[1]

Series B.—All Gravenstein apples except in the case of 6 (b), 8 (b), 9 (b) and 10 (b), where Yates apples were used.

Controls.

Distilled water.

(1) No browning, pits or signs of poisoning on any of the prepared spots.

(2) Light brown irregular pits beneath three contiguous prepared spots, possibly over a slightly bruised area, the other nine spots quite unaffected.

1. I could see distinct signs of poisoning on these apples, but as Dr. Rothera could not, they are given as doubtful. Lead nitrate rapidly destroys oxidase and penetrates slowly. Lead nitrate pits are always pale, copper sulphate ones much darker.

Mercuric chloride.

(3) 1 gram per 10,000 c.c. All the spots with brown pits 2-4 mm. deep.

(4) 1 gram per 100,000. All the spots with brown pits 1-3 mm. deep.

(5) 1 gram per 1,000,000. All the spots with brown pits 1-2 mm. deep.

(6) 1 gram per 100,000 in 3 % sodium chloride.

(a) Browned and invaded by Penicillium.

(b) Yates. Rather pale pits to all the prepared spots 1-2 mm. deep.

(7) 1 gram per 10,000 in 3 % sodium chloride. Large deep pits to all the prepared spots 4-6 mm. deep.[1]

(8) In 3 % sodium chloride alone.

(a) From superficial browning to pits ½ mm. deep.

(b) Yates. Superficial browning. No distinct pits.

Copper Sulphate.

(9) 1 per 10,000 in 3 % sodium chloride.

(a) Large well-defined pits 2-3 mm. deep, but paler than with copper sulphate alone.

(b) Yates. Large pits, but apple invaded by Penicillium through a bruise.

(10) 1 per 100,000 in 3 % sodium chloride.

(a) All the prepared spots browned, pits pale, barely exceeding 1 mm. deep.

(b) Yates. As above, but pits not exceeding 1 mm.

(11) 1 per 100,000 (copper sulphate alone).

Large dark pits to all the prepared spots 3-4 mm. deep.

These results closely coincide with those already published by me, and show that dilute metallic poisons produce browning and pit formation in the presence of isosmotic solutions of sodium chloride, the entry here taking place by diffusion only. Further, Gravenstein apples appear to resemble Five Crowns in their greater sensitivity as compared with Yates. In a pale-skinned apple it is difficult to be sure that the cut has not gone too deep, whereas in a red apple the fragments removed from the skin must show no colour. If they do, the cut has passed through the hypodermal layers. Further, in soft-fleshed apples some of the prepared spots may be

1 The mercuric chloride kills the cells before sufficient salt has entered to prevent browning.

made over slightly bruised areas, which will subsequently give an imitation of irregular pit formation. It was owing to the occasional unreliability of the controls that I abandoned in my first paper the use of pale-skinned, soft-fleshed apples. Even in such cases, however, an element of doubt only creeps in in determining the lowest limits of the poisonous concentrations. In the stronger solutions every prepared spot shows a brown pit which has a well-defined area centric to the prepared spot, and does not spread beyond a sharp boundary zone. If when using a soft-fleshed or pale-skinned apple, an occasional prepared spot on the control develops an apparent "poison" pit; this is usually a pale colour. If it is due to a bruise it will be irregular and not centric around the spot. If it is due to too deep a cut admitting micro-organisms it will slowly develop further in moist air, but the other prepared spots will be unaffected. If it is due to an invasion of fungal hypae, it will spread rapidly through the whole apple and the pulp will become soft and watery.

I might perhaps add that if red-skinned, hard-fleshed apples are used, this method forms the best possible class experiment to demonstrate—

(a) the indifference of the plant-cell to distilled water;
(b) the importance of the cuticle;
(c) the extreme sensitivity of the pulp cells to metallic poisons.

For class experiments the best solutions to use are 1 gram per 100,000 of mercuric chloride or copper sulphate, and the method can be used to some extent to test the freedom of distilled water from small amounts of soluble metallic poisons.